and pathology begins before or shortly after birth. These characteristics combined with normal fetal developmental properties including the small size, an immature immune system, and an abundance of proliferating progenitor cells in multiple organs highlight the potential of *in utero* gene therapy to treat select diseases. The development of safe, clinicallyrelevant prenatal delivery methods for gene therapy, including gene editing, is critical to the future application of *in utero* gene therapy. Lipid nanoparticles (LNPs) have emerged as an alternative, potentially safer, approach for nucleic acid delivery compared to traditional viral vector delivery. In this study, we use LNPs to prenatally deliver CRISPR technology in the mouse model of hereditary tyrosinemia type 1 (HT1) in which mutations in the *Fah* gene of the tyrosine catabolic pathway result in accumulation of upstream toxic metabolites and death by ~1 month of age. Specifically, we assess LNP-mediated delivery of adenine base editor (ABE) mRNA and SpCas9 mRNA to perform liver-directed gene editing to correct the *Fah* disease-causing mutation or silence the *Hpd* gene (to prevent accumulation of toxic metabolites) respectively and rescue the lethal phenotype. In an initial experiment, gestational day (E) 16 C57BL/6 fetuses were injected via vitelline vein with LNP containing GFP mRNA. Liver analyses by immunohistochemistry (IHC) and stereomicroscope 5 days post-injection demonstrated GFP expression supporting future LNP studies in the HT1 mouse model. *Fah-/-* fetuses were via the vitelline vein at E16 with LNPs containing either ABE mRNA and a *Fah*-targeting gRNA (LNP.ABE.Fah) or SpCas9 mRNA and an *Hpd*-targeting gRNA (LNP.SpCas9.Hpd). After birth, injected fetuses were cycled off NTBC with removal of the drug by 2 weeks of age. Fetuses injected with LNPs not containing gene editing RNA molecules served as controls. On-target editing efficiency in liver DNA at the time of sacrifice (at least 90 days after removal of NTBC) was assessed by next-generation sequencing. Phenotype correction was assessed by monitoring weight gain, survival at 90 days, and liver function tests. Finally, IHC of the liver was performed to assess FAH and HPD protein expression. On-target *Hpd and Fah* editing efficiencies in liver DNA 90 days after NTBC removal were 65.51+/-1.12% and 27.01+/-0.77% respectively. The high editing efficiency 90 days after removal of NTBC in both approaches is related to a known survival advantage conferred to edited cells. IHC analysis of experimental and control mice confirmed a reduction in HPD protein expression in mice injected with LNP.SpCas9.Hpd and increased FAH protein expression in recipients of LNP.ABE.Fah. Recipients of LNP.SpCas9.Hpd and LNP.ABE.Fah demonstrated liver function, weight gain and survival that was comparable to unedited *Fah-/-* mice maintained on NTBC and significantly improved compared to unedited *Fah-/-* mice in which NTBC was removed at 2 weeks of age. This study demonstrates the feasibility of using an mRNA-LNP delivery platform for therapeutic *in utero* adenine base editing and CRISPR-mediated nonhomologous end joining. It supports this approach as a treatment for HT1 and highlights its potential to treat other genetic liver diseases.

19. Correction of DMD Mutations in Human iPS-Derived Muscle Cells by Single-Cut CRISPR/Cas9-Based Gene Editing

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Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder caused by mutations in the *DMD* gene encoding dystrophin, a protein essential for sarcolemma integrity and stability. Among more than 4,000 mutations identified in patients with DMD, the majority are deletions that cluster in "hot spot" regions. Mutations that delete single or multiple exons of the dystrophin gene can be corrected by reframing or exon skipping to restore the dystrophin open reading frame. We previously showed that single-cut CRISPR/Cas9-based gene editing can induce exon reframing or skipping events thereby restoring the synthesis of near full-length dystrophin in the skeletal muscle, diaphragm, and heart (Min et al., 2019). To validate this strategy in human cells, we used induced pluripotent stem cells (iPSCs) from DMD patients carrying dystrophin out-offrame exon deletions and from corresponding siblings encoding wild type dystrophin (non-DMD). Corrected iPSC clonal lines were derived from DMD patient cells after gene editing using *Sp*Cas9 complexed with guide RNAs targeting exons 51 and 45 (generated in Dr. Olson's laboratory at UT Southwestern). iPS cells were then differentiated into cardiomyocytes (iCM) and skeletal muscle (iSKM) cells using previously established protocols (Chal et al., 2016; Lian et al., 2012). Beating iCMs and twitching iSKMs were observed as early as 6 and 15 days, respectively. Within 3-4 weeks of differentiation, cardiac (Troponin T) and skeletal muscle (Fast MyHCII) markers were observed in differentiated cells. As expected, no dystrophin was detected in iCMs or iSKMs derived from DMD iPSCs. However, restoration of dystrophin protein expression was evident in both iCMs and iSKMs derived from corrected iPSCs. Furthermore, similar to the non-DMD derived cells, dystrophin appeared to be correctly localized at the sarcolemma on skeletal muscle fibers in all edited clones. RTPCR and Sanger sequencing confirmed exon skipping or +1 nucleotide insertions (i.e. reframing events) in iCMs differentiated from edited clonal lines. In summary, our data provides an important proof of concept for efficient correction of *DMD* mutations and restoration of dystrophin expression in human DMD patient-derived iPSCs by CRISPR/Cas9-based gene editing. Furthermore, our strategy offers an attractive platform for the development of relevant *in vitro* DMD models and drug screening strategies.

20. Cell-Based Delivery Strategies for Artificial Transcription Factors in Preclinical Animal Models

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Therapies based on DNA-modifying proteins such as zinc finger, transcription activator-like effectors, and CRISPR/Cas9 to regulate gene expression are becoming viable strategies to treat genetically linked disorders through manipulation of endogenous gene regulation. The identification of an effective delivery systems for these proteins *in-vivo* remain a major translational hurdle. In this work, we evaluate a mesenchymal stem/stromal cell (MSC)based delivery system as a putative cell-based strategy for the secretion of DNA modifying proteins. MSCs are advantageous as a delivery system due to their favorable ease of culturing, immunomodulatory properties, and favorable clinical safety profile. Presently, we report the first the use of a Zinc Finger secreting MSC (ZF-MSC) in transgenic Angelman Syndrome (AS) mouse models.

In our *in-vivo* work we evaluate two routes of administration for ZFMSC - direct intracranial injection or access into the cerebral spinal fluid space. Secreted ZF protein from mouse ZF-MSC is detectable in the murine hippocampus 1-week following either intracranial or cisterna magna injection. This secreted ZF is able to activate the imprinted paternal Ube3a gene in a transgenic $Ube3a^{Yfp}$ reporter mouse at 1 and 3-weeks following either intracranial or cisterna magna injection of ZF-MSC. We detect high co-localization of secreted ZF protein within the CA1 and CA3 regions of the hippocampus in the $Ube3a^{Yfp}$ reporter mouse. ZF-MSC were detectable along cerebral spinal fluid rich regions such as the lateral ventricle, 3rd ventricle, and cerebellum following either route of administration. A significant increase in Yfp+ neurons are observable 1-week following intracranial ZF-MSC administration. A significant increase in $Ube3a^{Yfp}$ protein expression is observable in the hippocampus, midbrain, and cerebellum 3-weeks following a cisterna magna injection of ZF-MSC. An amelioration of motor deficits in rotarod and forepaw propulsion is observed 3-4 weeks following intracranial injection of ZF-MSC in the $Ube3a^{mat-/pat+}$ AS mouse. Overall the results of these studies demonstrate that ZF-MSC secrete functionally active ZF protein to activate paternal Ube3a in AS mouse models. This approach may provide a less-invasive, non-surgical means to deliver gene modifying therapies into the CNS through access of the cerebral spinal fluid injections.

21. A CRISPR-Mediated Strategy for *Mecp2* Gene Correction *In Vivo* as a New Treatment for Rett Syndrome

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Rett syndrome (RTT) is a severe neurological disorder caused by loss-of-function mutations in Methyl-CpG binding protein 2 (*MeCP2*) gene. Gene therapy strategies aimed to restore MeCP2 function hold great therapeutic potential since as shown in murine models, RTT phenotype can be reversed. Despite this, MeCP2 expression is not homogenous among different cell types and tight regulation of its expression is crucial to avoid adverse effects. Indeed, the overexpression of MeCP2 can lead to severe neurological alterations clinically comparable to RTT. In this scenario, a gene replacement approach, aimed to restore MeCP2 function under the control of the endogenous regulatory elements, represents an ideal strategy to avoid regulatory problems. Thus, we conceived a homology-independent CRISPR-based approach to introduce a minimal *Mecp2* coding sequence upstream of the endogenous translation start site. *In vitro* analyses on primary neuronal cultures revealed a high efficiency of *Mecp2* repair, with over 30% of neurons that re-activate Mecp2 expression under the control of the endogenous regulatory elements. Next, we exploited the AAVPHP.eB to validate the efficiency and the safety of this strategy *in vivo* in wild-type and symptomatic *Mecp2-null* mice. Molecular and phenotypic analyses confirmed the feasibility to repair in a site-specific manner *Mecp2* gene in the rodent brain, ameliorating the pathological phenotype of RTT mice without eliciting significant toxic reactions. This approach defines a novel therapeutic strategy with increased safety with respect to standard gene addition therapy strategies, holding important clinical implications for RTT.

Development of AAV Capsid Variants

22. A Novel Liver-Tropic AAV Capsid sL65 Shows Superior Transduction and Efficacy in Humanized Mice and Non-Human Primates

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Engineering the Adeno-associated virus (AAV) capsid facilitates the generation of recombinant vectors displaying novel properties of interest, including tropism, potency, ease of production and lower recognition by preexisting antibodies. The resulting vectors could target a broader patient population and be effective at reduced dosage, with lower manufacturing cost. In addition to using cutting-edge technology to create highly diverse capsid libraries, innovative screening methods must be used to validate the libraries and to successfully identify human-tropic capsids. Previously, the Children's Medical Research Institute reported the identification of novel capsid variants exhibiting high transduction

and expression levels in human hepatocytes using a model of humanized FRG mice. Here, we further characterized these lead capsids with regards to manufacturability, seroprevalence in a panel of 200 human sera samples, and expression efficacy as measured by human Factor IX (hFIX) production in non-human primates (NHPs). Our experiments led to the identification of a novel capsid with outstanding properties related to both manufacturability and in vivo potency. When used to package a recombinant AAV genome bearing human Factor IX, this capsid sL65 produces high titers ($7x10^{11}$ vg per mL of cell culture from suspension HEK293 cells). In NHPs (*Macaca fascicularis*), the hFIX expression level delivered by sL65 was up to 4-fold higher when compared to vectors pseudotyped with benchmark capsids AAV8 and LK03. There was a tight correlation between plasma hFIX concentration, episomal copy number and mRNA level in the liver, indicating that the higher potency was induced by increased transduction and expression compared to AAV8 and LK03 capsids. Biodistribution of vectors was investigated at 6 weeks post dosing by droplet digital PCR. A strict liver tropism was observed for sL65. Interestingly, in situ hybridization staining revealed that the vector distribution was uniform in the liver while the LK03 serotype showed preferential transduction in the periportal area. None of the animals exhibited any vector-associated adverse events. Finally, we found 58% of the samples in a panel of 200 human sera had neutralizing titers < 1:10 and 67% < 1:30. In summary, these data confirm the promising potential of capsid sL65 as a new vector for human liver. With the high potency in a humanized in vivo system and non-human primates, high production yield and low prevalence of pre-existing antibodies in the human population, sL65 is an excellent candidate to overcome the current limitations of traditional AAV vectors including toxicity caused by high dosage, high manufacturing costs and low translatability from mouse studies to human trials.

23. Efficient Design of Optimized AAV Capsids Using Multi-Property Machine Learning Models Trained across Cells, Organs and Species

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While next-gen high-throughput assays now enable us to learn how capsid sequence changes affect capsid functionality, measuring and optimizing capsid properties in the most therapeutically relevant models, such as non-human primates (NHP), remains challenging. The efficiency of transduction in target organs is often much lower than ideal, and most of the sequence space is non-functional. Therefore, the chance of identifying an improved variant through random search is miniscule. To overcome these challenges, we investigated to what extent machine learning models can improve the efficiency of AAV capsid design, as defined by the probability that a designed variant will have improved function. We synthesized and barcoded libraries containing 803,041 designed sequence variants of 3 natural AAV capsid serotypes and measured their properties as delivery vectors in cell culture, and in vivo in mice and NHPs. We show that single-property machine learning models trained on these data can improve the efficiency of library design by at least several hundred fold. Furthermore, we demonstrate the value of multi-property models in several ways. Models trained on multiple properties in combination help overcome data sparsity and measurement error, thereby improving model accuracy and providing a more reliable interpretation of experimental results. Multi-property models also provide a coherent framework in which to connect information from experiments across cell lines, organs, and species to the most relevant outcomes in NHP studies, thereby reducing the high resource and ethical burdens of NHP experimentation. Finally, multi-property models learn representations of the capsid landscape that can better optimize these vectors across the multiple properties that are key to enhanced therapeutic efficacy, for example: i) increasing transduction of target cells, ii) minimizing off-target tissue biodistribution, and iii) improving manufacturability. With further refinement, multi-property machine learning models will enable the design of highly optimized AAV capsids that open new frontiers in delivery, toward realizing the full potential of gene therapy.

24. Risk-Adjusted Selection for Validation of Sequences in AAV Design Using Composite Sampling

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High-throughput DNA synthesis methods have unlocked massive potential in the design of novel AAV vectors for gene therapy. New computational techniques are needed to generate candidate

sequences at the volume and quality necessary to take full advantage of these synthesis methods. Machine learning models trained on large datasets of AAV variants can predict the properties of unobserved sequences, allowing for screening of billions of candidates insilico before selecting the best ones for experimental validation. A naive approach to pruning billions of candidates down to hundreds of thousands for synthesis would involve selecting the top-scoring sequences according to machine learning model predictions. However, model predictions are never perfectly accurate, and a more principled approach can improve the chances of discovering high-performing variants. A similar problem arises later in the pipeline: promising candidates from high-throughput experiments are selected for medium- to lowthroughput validation by comparing their experimental measurements. Like machine learning models, experimental measurements are noisy and may be biased, and promising vectors from experiments in model organisms may not directly transfer to clinically valuable vectors in humans. Hedging against the risk of model and experimental error in this context is critical for improving the chances that the validation set includes the variants with the most desired ground-truth properties. Here we demonstrate the utility of a novel method, Composite Sampling (CS), that allows for pruning candidate sequences by accounting for model uncertainty and experimental noise, in a manner that maximizes the chances of including the best candidates in our validation set. CS is a tunable computational method that allows the experimenter to modulate validation risk when faced with noisy model or experimental evaluation. This method can be used in any setting where samples can be scored according to a desirability and a diversity metric. Here we show our method's value for AAV capsid design. We use retrospective studies in experimental AAV datasets and simulation of multiple synthetic datasets to demonstrate that Composite Sampling is consistently better than greedy approaches in selecting highperforming sequences for the validation set. In comparison to current approaches, our method increases the probability that high-throughput screens of capsid libraries will yield vectors that are optimized for therapeutic applications.

25. A Comparison of Methods Used for the Determination of Full and Empty rAAV Particles

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The use of recombinant Adeno-associated virus (rAAV) as a biological vehicle for the delivery of therapeutic DNA into humans provides an unparalleled treatment for a range of diseases. Although the production of therapeutic biologics for treating human diseases has been established, the first FDA approved gene therapy for human use occurred within recent years. To produce rAAV at the appropriate scale for treatment, requires effective manufacturing and purification of capsids containing the target DNA from the complex matrix of the upstream harvest. These processes may introduce undesirable process and product-related impurities that impact the overall performance and quality of the final drug product. One such product-related impurity is the presence of improperly packaged or "empty" (hereafter referred to as empty) rAAV capsids. The collective data indicate that the level of empty capsids in final products may reduce the *in vivo* efficacy and result in adverse events following administration. Regulatory guidance suggests that accurate analytical methods be used early in the product development cycle of gene therapies to evaluate and monitor the level of empty capsids. Sedimentation velocity analytical ultracentrifugation (SV-AUC) is considered a primary analytical method for the determination of full and empty rAAV particles. Additional methods have been utilized for the determination of the full empty ratio. These methods include transmission electron microscopy (TEM), vector genome to capsid determinations, and more recently cryogenic electron microscopy (Cryo-EM) and charge detection mass spectrometry (CD-MS). Each of these methods offers advantages and disadvantages in terms of assay sensitivity, accuracy, linearity, robustness, and utility for in-process testing. Presented here is the comparison of these methods (notably SV-AUC and CD-MS)

with an internally developed method to assess the full-to-empty ratio. A variety of parental and engineered proprietary capsids containing single-stranded and self-complementary vectors were assessed. Across multiple productions, SV-AUC, CD-MS, and our internal method were in good agreement for a variety of packaged DNA targets. SV-AUC and CD-MS are especially advantageous for the quantification of partially packaged DNA in rAAV, which tends to increase in abundance with self-complementary vectors. The internal method allowed for the timely measurement of full-to-empty particles at the cell lysate stage as well as purified material; however, the stage of sample purification as well as the packaged genome may impact which method is most suitable for determining levels of empty, partial, and full capsids. Results from this work suggest there is a benefit to using orthogonal methods to determine the level of empty particles.

26. A Tetracycline Enabled Self-Silencing Adenovirus (TESSA) Platform Delivers High-Quality, High-Titre, Multi-Serotype Recombinant Adeno-Associated Virus (AAV) Stocks

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A significant challenge for Adeno-associated virus (AAV) -mediated gene transfer has been the capacity to produce recombinant AAV vectors of high quality, at high-titre and at large scale. We have addressed these challenges by developing a proprietary TetracyclineEnabled Self-Silencing Adenovirus (TESSA) system that simultaneously provides Adenovirus (Ad) helper functions and encoded AAV Cap/ Rep. When cells carrying an AAV ITR-flanked transducing genome are infected with TESSA-Cap/Rep, high-titre rAAV stocks are produced that are essentially free of contaminating TESSA vector. We have now examined the ability of TESSA to

support production of adiverse range of AAV serotypes. The Capcoding region of AAV serotypes 1-9 were independently engineered into the E1 region of TESSA and the quality, physical and infectious titre of the rAAV produced by this system were characterised. For all rAAV serotypes, physical titres and infectious titres were invariably greater than that observed for conventional plasmid-based rAAV systems. For these serotypes, the ratio of full to empty particles is greatly improved. Moreover, for some AAV serotypes, the TESSA system delivered infectious titres 1000-fold greater than the plasmid-based helper-free systems. Importantly, TESSA-derived rAAV serotypes are competent for reporter gene and therapeutic gene transfer into a multiplicity of human cell types in vitro and a variety of model animal tissues in vivo. Therefore, we have developed a versatile system that can be applied to a range of naturally occurring AAV serotypes, and to experimentally engineered AAV capsids with novel tissue targeting phenotypes. The TESSA system provides unique opportunities for robust high-quality scale up of rAAV manufacture that will reduce costs and facilitate rapid progression to clinical application of novel rAAV-based gene therapies.

27. Next Generation AAV Drug Products: Enhanced Stability & Clinical Ease for High Titer Preparations

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Homology Medicines has developed a Plug-&-Play Process and Manufacturing Platform to develop and manufacture gene transfer and gene editing therapeutics for rare diseases, which utilizes our proprietary Clade F AAV capsids derived from hematopoietic stem cells (AAVHSCs). As our programs have progressed through development and into the clinic, we have increased focus on the Drug Product sciences which includes the thoughtful development of stabilizing formulations enabling high titers, easing of clinical storage and supply chains, and enhanced long-term stability. AAV preparations have long held a reputation as challenging not only for production, but for long-term stability at even low concentrations. However, here we demonstrate not only the stability of AAVHSCs in the liquid state, but also the impact of novel formulations on capsid stability. Our AAV preparations achieve titers in excess of 1E14 vg/mL and demonstrate stability for a minimum of one year at 2-8°C and more than six months at room temperature. Benefits of vector stability in the liquid state are a reduction in the need for -80°C-storage infrastructure and simplification of the clinical supply chain by enabling 2-8°C storage at the manufacturing site and clinical pharmacy. This work demonstrates the marked stability of our proprietary AAVHSCs as well as the impact of a well-developed formulation. Furthermore, this effort shows that by bringing a Drug Product focus to AAV product development, an organization can help support clinical and commercial success by providing long product expiries, smaller administration volumes, and 2-8°C supply chains.

28. Development of a Membrane-Based Affinity Matrix for Downstream Purification Process of Adeno-Associated Virus Vectors

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Adeno-associated virus (AAV) vectors are promising tools for gene therapy. Along with expanding market scale, reducing production costs is a key concern. Although the titer of AAV vectors in the upstream process has increased over the years, the downstream process is still outdated and should be improved. Affinity chromatography is a powerful tool for capture step of biologics production owing to its high selectivity. However, the chromatographic purification of AAV vectors from crude cell lysate is challenging due to high impurity contents in the loading material and damage to AAV transduction efficiency by contacting with low pH solution in the elution step. Tangential-flow filtration (TFF) is introduced prior to affinity chromatography to reduce the burden and to concentrate AAV vectors, but introduction of an additional process is ideally avoided to simplify the process and reduce production costs. Preventing damage from low pH denaturation increases overall AAV vector productivity in terms of a yield and quality. Herein, we developed a novel affinity matrix, which is composed of membrane-based matrix with a larger pore size than conventional beads resin. This enables a higher flow rate, resulting in a ten-fold shorter contact time than the conventional resin that compensates for the lack of the concentrating effect of TFF. This matrix provided a higher AAV vector yield in the elution pool than the beads resin owing to less contact with low pH conditions. Moreover, better cleanability was also shown likely due to less clogging inside the large pores. Our new affinity matrix intensifies the AAV vector affinity chromatography step and shows insight to develop a new platform of AAV vector downstream process.

Gene Therapy for Inborn Errors of Metabolism

29. Coadministration of AAV Expressing MDR3 (VTX-803) and ImmTOR Allows for Vector Re-Administration to Treat Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3) in Juvenile *Abcb4*$^{-/-}$ Mice

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Liver-directed AAV gene therapy has been shown to be an effective modality for the correction of genetic disorders, such as hemophilia, in adult patients. However, gene therapy for many inborn errors of metabolism, such as progressive familial intrahepatic cholestasis type 3 (PFIC3), would be most effective if administered in infancy or early childhood, to prevent irreversible damage. PFIC3 is a rare monogenic disease leading to cholestasis, cirrhosis and ultimately liver failure, with generally an early onset that requires early treatment when diagnosed in children under 3 years of age. PFIC3 is characterized by a dramatic reduction in biliary phosphatidylcholine (PC) content due to mutations in the *ABCB4* gene, which codes for multidrug resistance protein 3 (MDR3) and is responsible for transporting PC across the canaliculi membranes of hepatocytes into the bile, where PC neutralizes bile acid toxicity. A key challenge for AAV gene therapy in pediatric PFIC3 patients is the potential for therapeutic benefit to wane over time as the non-replicating AAV vector is diluted due to hepatic cell proliferation in the growing child, and that AAV cannot be re-administered due to the formation of high titers of persistent neutralizing antibodies. Here we tested the effectiveness of tolerogenic ImmTOR nanoparticles carrying the immunomodulating drug rapamycin to enable repeated intravenous administration of an hepatotropic AAV vector carrying human *ABCB4* cDNA (VTX-803) in 2-week-old juvenile *Abcb4*$^{-/-}$ mice when ImmTOR is co-administered with the first treatment of AAV. As a proof-of-concept study, we utilized a subtherapeutic dose of VTX-803 in order to focus on the experimental outcomes of coadministration with ImmTOR and readministration. VTX-803 when co-injected with ImmTOR allowed for a successful readministration of VTX-803 alone two weeks later and resulted in a robust and stable correction of the disease phenotype lasting over 7 months, while repeat dosing of the vector alone (at a sub-therapeutic dose) or together with empty SVP did not exhibit a therapeutic effect. In males, a temporary therapeutic effect was observed following only a single treatment of VTX-803 with ImmTOR, but not observed with empty SVP, suggesting that ImmTOR could increase vector transduction and/or transgene expression. This effect was observed through 10 weeks post-treatment, after which time the effect was lost, highlighting the importance of repeat dosing especially in juvenile animals with growing livers where transgene-positive cells can be diluted over time. Vector treatment without ImmTOR resulted in the production of AAV-specific neutralizing antibodies (NAbs), while vector coadministration with ImmTOR prevented NAbs production. Thus, this provides further evidence that redosing AAV gene therapy can be achieved through coadministration with ImmTOR, which has the dual benefit of potentially improving AAV vector transduction and mitigating vector-specific immunogenicity.

30. Preclinical Evaluation of Combined Adeno-Associated Virus and Nanoparticle Delivery of piggyBac® Transposon System for Durable Transgene Expression in the Growing Neonatal Murine Liver

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Gene delivery via recombinant adeno-associated virus (rAAV) has been shown to be efficacious in pre-clinical models and clinical trials for a variety of genetic diseases. A major limitation of current rAAVmediated gene therapy, however, is dilution of episomal rAAV and loss of transgene expression in rapidly dividing tissues as well as toxicity at higher rAAV doses. Loss of transgene expression and therapeutic efficacy are particularly limiting for gene therapy of infants and young children severely affected by metabolic and other disorders involving the liver. To address these issues, we have explored rAAV and/or novel nanoparticles (NP) as vectors for delivery of piggyBac® transposon and transposase to facilitate transgene integration in the host hepatocyte genome using ornithine transcarbamylase (OTC) deficiency as a disease model. A human OTC expression cassette in a piggyBac® transposon with liver-specific promoters was administered using rAAV with a liver-tropic capsid to neonatal wild-type and OTC deficient (Spf^{ash}) mice, with and without "Super" piggyBac (SPB), a hyperactive form of the transposase. As compared with transposon alone, concomitant rAAV delivery of SPB resulted in stable vector integration into the hepatocyte genome with durable and enhanced transgene expression for over 3 months according to vector copy number, bioluminescence imaging, IHC, human OTC mRNA and

protein levels and mitigation of the OTC disease phenotype. We further demonstrated that replacing rAAV delivery of SPB with a NP formulation for efficient liver delivery of mRNA resulted in similar high levels of durable transgene expression (~30% hepatocytes). Genomic modification and clonality of transposed hepatocytes are being further evaluated from LM-PCR and integration site analysis. These findings collectively demonstrate the unique potential of piggyBac® technology for *in vivo* liver-directed gene therapy for infants and young children, as well as the versatility afforded by utilizing viral and/or non-viral delivery systems for single-treatment and permanent correction of OTC deficiency and other genetic diseases.

31. Targeting Aberrant Acylation as a Novel Approach for Treating Methylmalonic Acidemia (MMA) and Related Other Organic Acidemias

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Organic acidemias (OAs), such as methylmalonic acidemia (MMA), are a group of clinically severe inborn errors of metabolism that typically arise from defects in the catabolism of amino- and fatty acids. The accretion of acyl-CoA species is postulated to cause intracellular toxicity and underlie the dysregulation of multiple intermediary pathways seen in the patients, such as the urea cycle and glycine cleavage system. Here, we explore an alternative pathophysiological consequence of impaired acyl-CoA metabolism: the accumulation of aberrant posttranslational modifications (PTMs) on enzymes in critical intracellular pathways. Using an MMA mouse model that recapitulates MMA-associated hepatic mitochondriopathy (Mmut-/-;TgMCKMmut), we surveyed PTMs in hepatic extracts with acyl-lysine antibodies and discovered widespread hyper-acylation. Next, we prepared affinity anti-acyl-lysine columns to enrich for modified proteins, and then performed mass spectrometry to characterize the PTM proteome. Excessive acylation of enzymes involved in glutathione, urea, arginine, tryptophan, valine, isoleucine, methionine, threonine, and fatty acid metabolism were detected in the MMA mice, and validated via immunoprecipitation analysis. We extended our analyses to other perturbed pathways, including the glycine cleavage system and mitochondrial replication, which we found to also be aberrantly modified in liver extracts from both MMA patients and Mmut-/-;TgMCKMmut mice as compared to respective controls The emerging pattern from our aggregate studies further supports a model where hyperacylation of key enzymes in pathways known to be dysregulated in MMA likely contributes to altered metabolism and identifies a new set of targets. With these new insights, we investigated the sirtuin (SIRT) family of enzymes as potential therapeutic agents given their known role as specialized deacylases. SIRT 1-7 were individually assayed for activity toward MMA specific acylations of modified protein substrate. An optimal SIRT emerged, but we noted that aberrant acylation also inhibited the functionality of our candidate enzyme, lowering enzymatic activity *in vitro.* Using rational mutagenesis, we created a "SuperSIRT" that was resistant to acylation-dependent inhibition, validated activity in vitro, cloned it behind a liver specific promoter (LSP), and packaged

with an AAV8 capsid. The resulting AAV8 LSP SuperSIRT or an AAV8 LSP EGFP control were then systemically delivered to juvenile Mmut-/-;TgMCKMmut and Mmut+/-;TgMCKMmut mice at a dose of $1e^{13}$ GC/kg, and followed by clinical, biochemical, and enzymatic analyses. After 1 month, the blood ammonia levels were significantly reduced in the treated mutant mice compared to the AAV8 LSP EGFP and untreated mutant control groups, while plasma methylmalonic acid levels remained unchanged. In hepatic extracts from the AAV8 LSP SuperSIRT treated Mmut-/-;TgMCKMmut mice, the aberrant acylation on key protein targets in the urea cycle and glycine cleavage pathway was reversed compared to GFP treated controls. In summary, our studies have identified a new PTM axis in patients and mice with MMA, which has allowed the development of a SuperSIRT gene therapy that could be used to treat all forms of MMA and might be extended to other disorders where aberrant acylation plays a role in disease pathophysiology, such as organic acidemias and fatty acid oxidation disorders.

32. AAV Liver Gene Therapy-Mediated Inhibition of FGF23 Signaling as a Therapeutic Strategy for X-linked Hypophosphatemia

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X-linked hypophosphatemia (XLH) is a rare disease due to increased fibroblast growth factor 23 (FGF23) secretion from bones which results in phosphate wasting in kidneys. Decreased circulating phosphate is the primary cause of severe skeletal deformities and short stature that greatly affect patients' quality of life. Conventional treatment for XLH requires life-long, repeated supplementation of phosphate and active vitamin D analogs and is associated with severe long-term side effects and poor compliance. Recently, the use of Burosumab (Crysvita®, Ultragenyx, and Kyowa Kirin), a monoclonal antibody for FGF23, has been proposed as an alternative treatment. Based on the central role of the overactive FGF23 pathway in the pathophysiology of FGF23, here we devised a liver-targeted AAV gene therapy strategy to inhibit FGF23 signaling and rescue bone pathology with a single injection. Secretion of an FGF23 competing factor (cFGF23) by the liver of a murine model of XLH led to the restoration of the impaired skeletal phenotype, significant reduction of osteomalacia and bone and joint alterations in Hyp-Duk mice. Our data provide proof-of-concept to the use of AAV liver gene therapy for the treatment of XLH, a prototypical disease associated to overexpression of soluble factors in tissues refractory to AAV gene therapy, thus expanding the reach of this therapeutic modality and providing novel options for the treatment of this disease category.

33. Comparison of Gene Addition Therapy in Genetically Distinct Mouse Models of Classical Phenylketonuria

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Phenylketonuria (PKU) is a highly complex biochemical disorder with nearly 1,000 pathogenic alleles in the phenylalanine hydroxylase (*PAH*) gene that cause a wide spectrum of effects on PAH enzyme dynamics. Clinical trials examining gene addition therapy for classical phenylketonuria (cPKU) have launched with much excitement, yet there is a lack of preclinical data on therapeutic outcomes in genetically distinct animal models. The longstanding cPKU $Pah^{enu2/enu2}$ mouse contains a missense mutation p.F263S (c.835 T > C), resulting in normal production of aberrant non-functional PAH. It has long been hypothesized that the mutant PAH monomers in this model may interfere with wildtype (WT) monomers, impairing overall function of the PAH holoenzyme complex, a phenomenon known as the dominant negative effect. Recently we created the $Pah^{dexon1/dexon1}$ cPKU mouse that contains a deletion of *Pah*

exon1 and is completely void of PAH protein, thus removing the variable of mutant monomer expression. In comparing overall PAH enzyme activity in heterozygous $Pah^{+/enu2}$ (N=6) and $Pah^{+/dexon1}$ (N=3) animals, we found that $Pah^{+/enu2}$ animals revealed markedly decreased enzyme activity (29 ± 5% WT activity) in comparison to $Pah^{+/dexon1}$ animals (58 ± 7% WT activity) with a $P < 0.0001$, and for the first time, indisputably confirmed the presence of a dominant negative effect caused by the mutant monomers produced from the Pah^{enu2} allele. This was further supported with liver-directed gene addition therapy studies comparing the two models. The murine liver-tropic adeno-associated virus serotype 8 was packaged with a Liver Specific Promoter driving expression of murine PAH (AAV2/8 LSPmPAH) and administered to 6-8 week old animals via retro-orbital injection that were harvested after two weeks for molecular analyses. All animals showed corrected blood Phe well below the therapeutic target range (below 360 μM), however they revealed stark differences in total PAH enzyme activity between $Pah^{enu2/enu2}$ and $Pah^{dexon1/dexon1}$ cPKU animals. Two $Pah^{enu2/enu2}$ cohorts of 3 males and 3 females (N=6 each) received medium dose (1×10^{11} vector genomes [vg]) or high dose (1×10^{12} vg) gene therapy. The total vector genomes per diploid liver genome (vg/dlg) ranged between 8-42 vg/dlg and 56-291 vg/ dlg, respectively, resulting in enzyme activity of 7.7 ± 2.2% and 14.8 ± 3.3%, respectively. In single $Pah^{dexon1/dexon1}$ animals, low dose (1×10^{10} vg), medium dose (1×10^{11} vg) and high dose (1×10^{12} vg) gene therapy conferred total liver vector genome copies of 2.9 vg/dlg, 16.2 vg/dlg and 50 vg/dlg, which resulted in an astonishing 4.8%, 42%, and 96.1% WT PAH enzyme activity. While more studies are needed to include more $Pah^{dexon1/dexon1}$ animals at all doses and $Pah^{enu2/enu2}$ animals at low doses, this data reveals the profound impacts genotype can have on overall enzyme dynamics in the setting of gene addition therapy for PKU. A further advantage of the $Pah^{dexon1/dexon1}$ cPKU animal model for gene therapy development is the ability to perform immune-based molecular analyses to localize and quantify gene therapy delivered PAH enzyme, a feat that is impossible in the $Pah^{enu2/enu2}$ model. Liver immunohistology of gene therapy treated $Pah^{dexon1/dexon1}$ animals revealed a dose dependent expression of PAH, which reveals critical knowledge that could be used to further optimize gene therapy development for PKU.

34. AAV8 Gene Therapy as a Potential Treatment in Adults with Late-Onset Ornithine Transcarbamylase (OTC) Deficiency: Updated Results from a Phase 1/2 Clinical Trial

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Introduction: OTC deficiency is an X-linked urea cycle disorder resulting in episodic hyperammonemia that can cause cumulative neurocognitive damage and even death. The current standard of care includes a proteinrestricted diet and nitrogen-scavenging agents, but there remains high unmet medical need with continued risk of hyperammonemic crises. DTX301, an AAV8 vector containing the *OTC* transgene, is currently under investigation for the treatment of OTC deficiency. **Methods:** CAPtivate (NCT02991144) is an ongoing global, multicenter, open-label phase 1/2 dose-escalation trial evaluating the safety and preliminary efficacy of DTX301 in adults with late-onset OTC deficiency. The primary endpoint is incidence of adverse events (AEs). Secondary endpoints are changes in the rate of ureagenesis and 24-hour plasma ammonia levels. Patients received a single IV infusion of DTX301 at the following doses: Cohort 1 (2×10^{12} Genome Copies [GC]/kg), Cohort 2 (6×10^{12} GC/kg), and Cohort 3 (1×10^{13} GC/kg). Study duration is 52 weeks followed by up to 4 years of long-term follow up. A partial responder is defined as a patient with a clinically meaningful and sustained increase in rate of ureagenesis with stabilization or improvement in ammonia control. A complete responder is defined as a patient who has also successfully discontinued ammonia-scavenging drugs and protein-restricted diet. **Results:** DTX301 dosing of 3 patients in each of cohorts 1, 2, and 3 is complete. Cohort 4 (1×10^{13} GC/kg with prophylactic oral steroid taper) is enrolling. No treatment-related serious AEs or dose-limiting toxicities were reported; all AEs were mild or moderate (grade 1, 2) during the study. Seven patients experienced treatment-emergent AEs (TEAEs) that were considered related to study drug. Five patients experienced asymptomatic ALT increases consistent with those seen in other AAV gene transfer clinical trials. ALT increases were managed and resolved with a protocol-specified tapering regimen of oral corticosteroids administered in outpatient setting. Other TEAEs considered related to study drug were photophobia, headache, hypertension, vector-induced hepatitis, and hypophosphatemia. Overall, 6 of 9 patients responded to DTX301: 3 patients were complete responders, and 3 patients were partial responders. All 9 treated patients maintained or improved ammonia control. Cohort 1 had one complete responder. Cohort 2 had 1 complete responder and 1 partial responder. Cohort 3 had 1 complete responder and 2 partial responders. The longest-treated responders from cohorts 1 and 2 are showing a durable response at 2.5 to 3 years after treatment and remain clinically and metabolically stable with good ammonia control. **Conclusions:** Data from CAPtivate indicate that DTX301 has an acceptable safety profile and may be a potential new therapy with longterm therapeutic benefit for patients with OTC deficiency. Followup of all patients is ongoing and enrollment in cohort 4 is nearly complete.

35. AAV-Mediated Delivery of MiRNA-34B/C Improves Liver Fibrosis

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Liver fibrosis is a major complication of chronic liver diseases and is orchestrated by an intricate molecular network. MiRNAs have been known to regulate several distinct processes involved in liver fibrosis. In this study, we found a novel miRNA cluster, including miR-34b and -c, to be upregulated in several forms of liver fibrosis both genetic and acquired. We found that murine hepatic miR-34b/c is upregulated in liver diseases due to ABCB4 deficiency and α-1 antitrypsin deficiency, disorders prone to the development of liver fibrosis. miR-34b/c upregulation was also found to occur in mouse models of liver fibrosis induced by thioacetamide and CCl_4. Mechanistically, miR-34b/c expression was dependent upon JNK-mediated phosphorylation of FOXO3 transcription factor on Ser574. Deletion of miR-34b/c resulted in early development of liver fibrosis and increased signaling of PDGF, a target of miR-34b/c. Moreover, miR-34b/c was effective in blunting TGF-β-mediated activation of human hepatic stellate cells, a key event in liver fibrosis development. Finally, we found that AAVmediated hepatocyte-specific overexpression of miR-34b/c significantly ameliorated liver fibrosis in two independent mouse models of acquired liver fibrosis (thioacetamide and CCl_4).In conclusion, this study reveals a novel pathway involved in liver fibrosis that is potentially implicated in both genetic and acquired forms of hepatic fibrosis. Furthermore, it supports miR-34b/c as a novel therapy against liver fibrosis.

Genetic Blood and Immune Disorders

Genetic Blood and Immune Disorders

36. Follow-Up of a Phase I/II Gene Therapy Trial in Patients with Fanconi Anemia, Subtype A

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Fanconi anemia (FA) is a monogenic inherited disorder mainly characterized by congenital abnormalities, childhood bone marrow failure (BMF) and cancer predisposition. Here we report the results of the 1-3 year follow-up of the eight evaluable FA-A patients corresponding to a phase I/II gene therapy trial. $CD34^+$ cells were mobilized with filgrastim and plerifaxor and collected following 2-3 apheresis procedures. $CD34^+$ cell-enriched fractions were transduced during a total period of 20-24h with the PGK-FANCA.Wpre* lentiviral vector and then infused without any pre-conditioning regimen. Nine patients age 3-7 years were infused with a range of $7.3x10^4$ to $1.9x10^6$ $CD34^+$ cells/kg. One patient was withdrawn from the clinical trial due to contamination of the medicinal product and bacteremia which was treated by antimicrobial therapy. Vector copy numbers (VCN) in colonies derived from the manufacturing products ranged from 0.2 to 0.9 VCN/cell. VCNs in patients' PB and BM after infusion of transduced cells showed slow but progressive engraftment of genecorrected cells in six out of the eight evaluable patients, reaching stabilized values as high as 0.6 copies/cell through the end of the follow-up (3 years). Insertion site studies confirmed the safety of the LV with 1-3 years follow-up. VCN increases were associated both with a progressive MMC-resistance of BM progenitor cells and a reduction in the chromosomal instability in PB T cells exposed to diepoxybutane. Stabilized, and even improved PB cell counts have been observed in patients with higher levels of gene correction. Nevertheless, two out of the eight evaluable patients who were treated at advanced stages of the disease or infused with low numbers of corrected $CD34^+$ cells showed progressive evolution of BMF. Our results demonstrate that gene therapy of non-conditioned FA patients has the potential to prevent BMF, and support the infusion of corrected $CD34^+$ cells prior to the development of BMF. Based on these results, a global phase II clinical trial is currently ongoing under the sponsorship of Rocket Pharmaceuticals Inc., focused on the prevention of BMF by means of the infusion of higher numbers of corrected $CD34^+$ cells in early stages of the disease.

37. Liver Gene Therapy with Lentiviral Vectors Corrects Hemophilia A in Mice and

Achieves Normal-Range Factor VIII Activity in Non-Human Primates

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Liver gene therapy with adeno-associated viral (AAV) vectors delivering a clotting factor transgene into hepatocytes has shown multiyear therapeutic benefit in adults with hemophilia. However, the mostly episomal nature of AAV vectors currently challenges application of AAV-vector mediated liver gene therapy to young pediatric patients. In contrast, lentiviral vectors (LV) integrate into the target cell chromatin and are maintained as cells divide. We previously developed LV that achieve stable and therapeutic levels of coagulation factor IX (FIX) transgene expression in the liver of adult mice, dogs and non-human primates (NHP) after systemic delivery. To evaluate LV-mediated liver gene therapy for hemophilia A, we generated LV expressing engineered versions of the human B-domain deleted Factor VIII (FVIII) by codon optimization of the transgene (coFVIII) and the inclusion of a nonstructured XTEN polypeptide (coFVIII.XTEN), known to increase the half-life and secretion of the payload protein, in the B-domain region. We administered LV expressing FVIII, coFVIII or coFVIII. XTEN intravenously (i.v.) to newborn hemophilia A mice and observed long term FVIII activity up to 200% of normal and restoration of hemostasis in mice treated with LV encoding for engineered transgenes, with transgene output 10-20 fold higher for coFVIII compared to FVIII transgene and 10-fold higher for coFVIII.XTEN than coFVIII. We then set out to evaluate FVIII expression in NHP and produced large-scale batches of allo-antigen free and phagocytosis shielded (CD47 high) LV carrying coFVIII or coFVIII.XTEN. We administered 1e9 transducing units (TU)/Kg (n=2) or 3e9 TU/Kg (n=3) dose for LV.coFVIII.XTEN or 3e9 TU/Kg (n=2) or 6e9 TU/Kg (n=3) dose for LV.coFVIII. A corticosteroid immune-suppression regimen was applied from day -1/3 to day +7/9, since human FVIII is known to be highly immunogenic in NHP. Administration of LV via peripheral vein was well tolerated with no significant changes in body temperature. Selflimiting leukopenia and limited serum aspartate aminotransferases (AST) elevation were observed. Therapeutically relevant FVIII amounts were observed in all treated animals, with the target 60-100% of normal human FVIII activity achieved at 3e9 TU/Kg dose for the LV.coFVIII. XTEN treatment group. We monitored both anti-FVIII antibody (Abs) formation, acute cytokine response to LV administration and T cell responses in the blood and the spleen of treated NHP. Upon corticosteroids discontinuation, all NHP developed anti-FVIII Abs, but 4/5 LV.coFVIII.XTEN treated NHP maintained LV-positive hepatocytes in the liver at the end of the study and their splenocytes did not respond to *ex vivo* FVIII stimulation. On the contrary, only 1/5 LV.coFVIII treated NHP maintained LV-positive hepatocytes in the liver and all of them showed splenocyte activation after FVIII stimulation *ex vivo*. Overall, our data show efficient and well tolerated gene transfer to the liver of NHP by LV, with an improved therapeutic index for the engineered FVIII.XTEN transgene, supporting further pre-clinical and potentially clinical development of this gene therapy strategy.

38. Towards Clinical Translation of Hematopoietic Cell Gene Editing for Treating Hyper-IgM Type 1

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HIGM1 is caused by mutations of CD40L, whose absence in CD4 T cells impairs signaling for B cell activation and Ig class-switching. Since unregulated CD40L expression leads to lymphoproliferation/ lymphoma, gene correction must preserve its physiological regulation. Gene editing of either autologous T cells or hematopoietic stem cells (HSC) held promise for treating HIGM1. We developed a “one size fits all” editing strategy to insert a 5’-truncated corrective CD40L cDNA in the first intron of the native human gene, effectively making expression conditional to targeted insertion in the intended locus. By exploiting a protocol that preserves T stem memory cells (TSCM), we reproducibly obtained ~40% of editing efficiency in healthy donor and patients derived T cells, restoring regulated, although partial, CD40L surface expression that was sufficient to restore helper function on B cell co-cultures. To select, track and potentially deplete edited T cells, we coupled the corrective cDNA with a clinically compatible selector gene and confirmed that enriched T cells preserved their engraftment capacity in NSG mice. Unexpectedly, the presence of an IRES-linked downstream coding frame counteracted the shorter half-life of transcript from the edited locus, allowing replenishment of intracellular stores and surface translocation of physiological amounts of CD40L upon activation. We also tailored the *CD40L* editing strategy to human HSC, reaching up to 15-30% editing in HSC long term engrafting NSG mice, depending on the HSC source. We then modelled the therapeutic potential of both T cell and HSC gene therapy by infusing increasing proportions of WT murine cells, as surrogates of edited cells, in HIGM1 mice. Administration of functional T cells at clinically relevant doses in HIGM1 mice, preconditioned or not with different lymphodepleting regimens, achieved long term stable T cell engraftment and partial rescue of antigen specific IgG response and germinal center formation in splenic follicles after vaccination with a thymus dependent antigen.

Remarkably, infusion of T cells from mice pre-exposed to the antigen, mimicking treatment of chronically infected patients, was